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In vivo imaging and quantification of regional adiposity in zebrafish

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Abstract

Adipose tissues (ATs) are lipid-rich structures that supply and sequester energy-dense lipid in response to the energy status of an organism. As such, ATs provide an organism energetic insurance during periods of adverse physiological burden. ATs are deposited in diverse anatomical locations, and excessive accumulation of particular regional ATs modulates disease risk. Therefore, a model system that facilitates the visualization and quantification of regional adiposity holds significant biomedical promise. The zebrafish (*Danio rerio*) has emerged as a new model system for AT research in which the entire complement of regional ATs can be imaged and quantified in live individuals. Here we present detailed methods for labeling adipocytes in live zebrafish using fluorescent lipophilic dyes, and for identifying and quantifying regional zebrafish ATs.

INTRODUCTION

The prevalence of obesity and overweight has reached epidemic proportions worldwide (Yach, Stuckler, & Brownell, 2006). Obesity is recognized as a major risk factor for the development of insulin resistance, type II diabetes, nonalcoholic fatty liver disease, and cardiovascular disease (Morse, Gulati, & Reisin, 2010). Therefore, understanding how obesity increases disease risk is a primary research question and of central global public health concern.

Obesity develops when energy intake exceeds energy expenditure, resulting in increased storage of excess energy, in the form of triacylglycerides (TG), within adipose tissues (ATs) (Rosen & Spiegelman, 2006). The major cellular constituent of ATs is lipid-rich adipocytes (or “fat cells”). ATs have traditionally been classified into white AT-containing large, lipid-filled adipocytes and thermogenic brown AT-containing smaller, multilocular adipocytes (Berry, Jiang, & Graff, 2016; Peirce, Carobbio, & Vidal-Puig, 2014). Zebrafish and other ray-finned fishes are not considered to possess brown AT; therefore, we will not discuss brown AT further in this chapter. White adipocytes (hereafter referred to as adipocytes), within AT, store surplus energy in the form of TG and hydrolyze accumulated TG for use as a fuel source during times of nutrient deprivation (Redinger, 2009). Adipocytes are present

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in bony vertebrates (e.g., fish, amphibians, reptiles, birds, and mammals), and AT is considered the primary site of energy storage in these vertebrate taxa (Gesta et al., 2006). White adipocytes exhibit a unique morphology and routinely grow to sizes >100 μm in diameter due to stored TG within single, large lipid droplets (LDs) (Farese & Walther, 2009). This unique cellular morphology greatly aids the identification of adipocytes.

Mammalian in vivo and in vitro cell culture systems have contributed the majority of current knowledge on AT cell biology (Virtue & Vidal-Puig, 2010). However, the importance of AT to commercial aquaculture, and the increasing use of zebrafish as a biomedical model system, has provided a rapidly expanding knowledge base on AT in teleost fish (the largest subclass of Actinopterygii or ray-finned fish). Many teleost species analyzed to date accumulate lipid within AT (Bou et al., 2016; Flynn, Trent, & Rawls, 2009; Imrie & Sadler, 2010; Johansson, Morgenroth, Einarsdottir, Gong, & Bjornsson, 2016; Song & Cone, 2007). Further, deposition and mobilization of lipid within teleost AT are altered in response to nutritional manipulation, suggesting the energy storage functions of AT are conserved between teleosts and mammals (Albalat et al., 2007; Bou et al., 2014; Flynn et al., 2009; Imrie & Sadler, 2010; Salmeron et al., 2015). Furthermore, histological analysis reveals evolutionarily conserved morphological features of teleost adipocytes, including large cytoplasmic LDs, caveolae, and close association with capillaries (Flynn et al., 2009; Imrie & Sadler, 2010; McMenamin, Minchin, Gordon, Rawls, & Parichy, 2013; Minchin et al., 2015). In addition, teleost adipocytes express genes associated with adipocyte differentiation (*fatty acid binding protein 11a*, *fabp11a*; *peroxisome proliferator-activated receptor gamma*, *pparg*; and *CCAAT/enhancer binding protein alpha*, *cebpa*) (Flynn et al., 2009; Ibabe, Bilbao, & Cajaraville, 2005; Imrie & Sadler, 2010; Oku & Umino, 2008; Vegusdal, Sundvold, Gjoen, & Ruyter, 2003), adipocyte lipolysis (*lipoprotein lipase*, *lpl*) (Oku, Tokuda, Okumura, & Umino, 2006), and adipocyte endocrine function (*leptin*, *lep*, *adiponectin*, *acrp30*, and *adipsin*, *cfp*) (Imrie & Sadler, 2010; Michel, Page-McCaw, Chen, & Cone, 2016; Vegusdal et al., 2003). Homologous to mammals, fish AT also possesses a stromal vascular fraction (defined as a heterogeneous population of stromal cells isolated by enzymatic digestion of AT), which contains adipocyte progenitors (Rodeheffer, Birsoy, & Friedman, 2008; Tang et al., 2008; Todorovic, Skugor, Krasnov, & Ruyter, 2010; Vegusdal et al., 2003). Together, the considerable functional, morphological, and molecular homology between teleost and mammalian ATs suggests new insights into AT biology gained in the zebrafish system will be directly translatable to humans and other vertebrates.

AT is not a single homogenous tissue in mammals and fishes and is distributed at specific anatomical locations throughout the body (Fig. 1). In humans, the largest sites of AT deposition are either subcutaneous (defined as between muscle and skin) or intra-abdominal (within the abdominal cavity) (Gesta et al., 2006; Karpe & Pinnick, 2015; Shen et al., 2003). Anatomically distinct depots display different molecular and physiological characteristics (Gesta et al., 2006; Peinado et al., 2010; Vidal, 2001; Vohl et al., 2004) and have different risk associations for obesity-related disorders (Kissebah & Krakower, 1994). In particular, visceral AT (VAT, intraabdominal AT surrounding internal organs) is associated with more adverse risk factors than subcutaneous AT (Despres, 1998; Fox et al., 2007). Teleost AT is also deposited in both subcutaneous and intra-abdominal positions, including VAT locations, raising the possibility that developmental programs responsible for AT anatomy have been

maintained during vertebrate evolution (Flynn et al., 2009; Imrie & Sadler, 2010; McMenamin et al., 2013; Minchin et al., 2015; Weil, Sabin, Bugeon, Paboeuf, & Lefevre, 2009). Further, recent work from our lab suggested that the control of AT distribution may have a conserved molecular basis between humans and fish (Minchin et al., 2015). However, the molecular and cellular basis for AT regionalization and disease risk associations is still largely unknown. Thus, the development of a novel model that facilitates the investigation of regional adiposity within whole animals is desperately needed.

1. RATIONALE

Current analysis of AT is predominantly conducted after fixation and histological sectioning of ATs. This often results in incomplete preservation of AT architecture and limited information on cellular interactions and dynamics (Xue, Lim, Brakenhielm, & Cao, 2010). In addition, imaging of whole animal AT deposition in mammals is technically challenging, is typically restricted to low resolution views, and has only been undertaken on a limited scale (Shen & Chen, 2008). Moreover, most of our knowledge of mammalian ATs is derived from adult stages due in part to the difficulty of accessing ATs during the gestational stages when they initially develop (Ailhaud, Grimaldi, & Negrel, 1992). As a consequence, outstanding questions regarding the spatial and temporal dynamics of *in vivo* AT formation and growth remain understudied. Innovative approaches have been developed to address these gaps in our knowledge, such as high-resolution imaging of resected AT cultured *in vitro* (Nishimura et al., 2007) and *in vivo* imaging of adipocyte precursors introduced into mice fitted with an implanted cover slip (Nishimura et al., 2008). However, these approaches do not permit imaging of ATs within the intact physiological context of a living organism. Mathematical modeling has also been used to predict *in vivo* mechanisms of AT growth, but these models remain largely untested due to a paucity of suitable *in vivo* model systems (Jo et al., 2009). There is therefore a pressing need for new experimental platforms for image analysis of AT formation and function in live animals.

The features of the zebrafish system are especially well suited to meet these needs. Zebrafish develop externally and are optically transparent from fertilization through the onset of adulthood, permitting *in vivo* imaging of dynamic cellular events during AT formation and growth (Fig. 1) (Flynn et al., 2009; Minchin et al., 2015). This provides new opportunities to investigate the earliest stages of AT morphogenesis, a process poorly understood in mammals with potentially high relevance for obesity and metabolic disease. The small size of the zebrafish also facilitates whole animal imaging of multiple adipose depots, unlike mammalian systems in which specific adipose depots are difficult to access (Fig. 1) (McMenamin et al., 2013; Minchin et al., 2015). Real-time imaging of living ATs is also possible in the zebrafish, enabling observation of molecular and cellular events over short time scales (Flynn et al., 2009; McMenamin et al., 2013). Furthermore, the amenability of the zebrafish to *in vivo* imaging permits longitudinal imaging of AT in individual animals, which can be used to mitigate complications from interindividual variation in adiposity (Flynn et al., 2009; McMenamin et al., 2013). As described earlier, the identification of extensive conserved homologies between teleost and mammalian AT suggests that insights gained in the zebrafish system could be applicable to humans and other vertebrates.

These diverse imaging strategies require robust methods for labeling the cellular constituents of AT in live animals. In this chapter, we present methods for labeling adipocytes in zebrafish using fluorescent lipophilic dyes (FLDs) that specifically incorporate into adipocyte LDs, for imaging ATs in live zebrafish using stereomicroscopy and guidelines on assessing the regional composition of zebrafish ATs.

2. MATERIALS

- Adult zebrafish. Any strain of adult zebrafish can be used for this protocol. Zebrafish lines may be obtained from the Zebrafish International Resource Center (ZIRC). All experiments should be performed in accordance with protocols approved by the user's Institutional Animal Care and Use Committee.
- Large nets (Aquatic Ecosystems, cat. no. AN8).
- Zebrafish aquarium (system) water.
- Breeding tanks (Laboratory Product Sales, cat. no. T233792).
- Plastic tea strainer, 7 cm (Comet Plastics, cat. no. strainer 0).
- Scienceware pipette pump (Fisher Scientific, cat. no. 13-683C).
- Wide-bore Pasteur pipettes (Kimble Chase, cat. no. 63A53WT).
- 100× 15 mm Petri dishes (Fisher Scientific, cat. no. 0875712).
- Methylene blue stock solution (0.01%) (Sigma, cat. no. M9140). Dissolve 50 mg methylene blue in 500 mL dH₂O. Dilute this stock solution 1:200 in fresh zebrafish aquarium system water to prevent growth of bacteria and mold during embryonic development.
- Distilled water (dH₂O).
- Fluorescence stereomicroscope (e.g., Leica MZ 16F or M205 FA) equipped with an eyepiece graticule and the following Leica emission filter sets: GFP2 (510LP) for the green FLDs (i.e., BODIPY 505/515, 500/510, NBT-Cholesterol, BODIPY FL C5 and the yellow-orange dye, Nile Red); YFP (535-630BP) for the yellow, orange, and orange-red dyes (i.e., BODIPY 530/550, 558/568, and Cholesteryl BODIPY 576/589); and Texas Red (610LP) for HCS LipidTOX Red/Deep Red. See Table 1 for a full description of FLDs. Equivalent fluorescence stereomicroscopes and filter sets can be used from alternative manufacturers.
- Air incubator set at 28.5°C (Powers Scientific Inc., cat. no. IS33SD).
- 2-L fish tanks (Marine Biotech, cat. no. 10198-00A).
- Assorted mesh drainage plugs for 2-L fish tanks (Marine Biotech, 425 µm, cat. no. 10222-01A; 600 µm, cat. no. 10222-02A; 1600 µm, cat. no. 10222-03A; 4000 µm, cat. no. 10222-04A).
- Brine shrimp (*Artemia franciscana*) cysts (Utah strain; Aquafauna Bio-Marine Inc., cat. no. ABMGSL-TIN90). Detailed brine shrimp hatchery methods are

included in *The Zebrafish Book* (Westerfield, 1995). Briefly, 80 mL of brine shrimp cysts are momentarily immersed in bleach before rinsing with system water. After rinsing, the cysts are added to 12 L of system water supplemented with 10 g sodium bicarbonate and 155 g sodium chloride. The cysts are aerated vigorously for 24 h, under continuous light. The hatched brine shrimp are filtered through a 105- μ m mesh sieve and diluted in 2 L of system water. Although brine shrimp hatching rates can vary, we typically find this procedure generates $\sim 4 \times 10^7$ brine shrimp per 24 h.

- Sodium bicarbonate (Aquatic Ecosystems, cat. no. SC12).
- Sodium chloride (Fisher Scientific, cat. no. S96860).
- Brine shrimp net (Aquatic Ecosystems, cat. no. BSN1).
- 15-mL conical tubes (polystyrene or polypropylene) (Becton Dickinson, cat. no. 35-2099).
- Plastic transfer pipettes (Samco Scientific, cat. no. 225).
- FLDs (see Table 1 for details). Chloroform:MeOH (2:1) is typically used as a solvent when making stock solutions of lipophilic dyes. However, chloroform:MeOH cannot be added directly to system water containing zebrafish. Therefore, the desired quantity of chloroform:MeOH stock solution containing lipophilic dye is air dried in a 1.6-mL microcentrifuge tube for ~ 10 min before being resuspended in 10 μ L of 100% EtOH. The 10 μ L of 100% EtOH can be added directly to system water containing zebrafish. Alternatively, dimethyl sulfoxide (DMSO) or acetone can be used as solvents when making stock solutions, and can be added directly to system water containing zebrafish. However, use of DMSO and acetone as solvents is not advised as the resulting stock solution is less stable over long periods of storage. Stock solutions of FLDs are kept in the dark at -20°C .
- Chloroform (Fisher Scientific, cat. no. BP1145-1).
- Methanol (MeOH) (VWR, cat. no. BDH1135-4LP).
- Ethanol (EtOH) (Decon Labs, Inc., cat. no. 2716).
- DMSO (Fisher Scientific, cat. no. D128-1).
- Acetone (Mallinckrodt Chemicals, cat. no. 2440-02).
- Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine or MS222) stock solution (24 $\times$) (Sigma, cat. no. A5040-110G). Combine 0.8 g of tricaine, 4.2 mL of 1 M Tris pH 9.0, and 195.8 mL of dH₂O. Adjust pH to between 7.0 and 7.5, and store at 4°C . Anesthetizing concentration is 1 $\times$, and euthanizing concentration is 5 $\times$.
- Methyl cellulose (4%) (Sigma, cat. no. MO387-100G). Dissolve 4 g methyl cellulose in 100 mL dH₂O. Make 1 mL aliquots in 1.6 mL microcentrifuge tubes and freeze at -20°C .

- Low melting point (LMP) agarose (1%) (Fisher Scientific, cat. no. BP165-25). Dissolve 1 g LMP agarose in 100 mL of 1× phosphate buffered saline (PBS). Make 1 mL aliquots in 1.6 mL microcentrifuge tubes and freeze at -20°C .
- 1.6-mL microcentrifuge tubes (Genesee Scientific, cat. no. 22-282A).
- Heat block set at 65 and 42°C (Denville Scientific, Inc., D1100).
- 24-well plastic culture plates (Greiner Bio-one, cat. no. 662160).
- Metal dissection probe (Fine Science Tools, cat. no. 10140-01).
- 30-mm Petri dish with glass cover slip as base (MatTek Corp., cat. no. P35G-1.5-10-C).
- Epinephrine stock solution (100 mg/mL) (Sigma, cat. no. E4375-5G). Dissolve 1 g epinephrine powder in 10 mL of dH_2O . Store at 4°C in the dark. Add 3 mL of stock solution to 30 mL system water (10 mg/mL final concentration) containing fish for 5 min to contract melanosomes (Rawls & Johnson, 2003).
- Paraformaldehyde (PFA) stock solution (4%) (Acros, cat. no. 30525-89-4). Dissolve 4 g PFA powder in 99 mL prewarmed 1× PBS. Once cooled, add 1 mL of DMSO.
- PBS stock solution (25×). Dissolve 200 g sodium chloride, 5 g potassium chloride, 36 g sodium dihydrogen phosphate, and 6 g monopotassium phosphate in 1 L dH_2O and autoclave.
- Tween-20 (Fisher Scientific, cat. no. BP337-500).
- Laser scanning confocal microscope (e.g., Zeiss LSM 510), equipped with Argon 488 nm, HeNe1 543 nm, and HeNe2 633 nm excitation lasers. Other platforms, such as light-sheet, spinning disk confocal, and multiphoton fluorescence microscopes, can also be used.

3. METHODS

3.1 OBTAINING ZEBRAFISH EMBRYOS

1. [Day 1, duration ~60 min] The day before embryos are required, use a large net to place suitable breeding pairs of adult zebrafish in specialized breeding tanks filled with fresh system water. Adults remain in breeding tanks overnight and typically spawn once the aquarium lights turn on the following morning. Be sure to label each breeding tank with the genotype and stock number(s) of the respective breeding pair.

Optional: Specific fluorescent lipid probes can be used in conjunction with transgenic zebrafish lines expressing fluorescent proteins (FPs) to facilitate cell localization studies (see Table 1 for excitation/emission properties of lipid probes). If required, obtain transgenic zebrafish from ZIRC (see Section 2).

2. [Day 2, duration ~60 min] To collect fertilized embryos, remove adult zebrafish to a different tank. Maintain labeling system of adult fish to track parentage of embryos. Collect embryos by pouring through a tea strainer. Clean embryos by rinsing multiple times in fresh system water, and dispense groups of 20–40 embryos into each 100-mm Petri dish filled with 30 mL of fresh system water. View embryos on a light stereomicroscope and remove unfertilized embryos using a wide-bore Pasteur pipette and pipette pump. Place fertilized embryos within an air incubator at 28.5°C.

Optional: If natural breeding is unsuccessful, embryos can alternatively be generated by in vitro fertilization (or “squeezing”) using established protocols (Westerfield, 1995).

Optional: Methylene blue stock solution can be added to system water (0.01% final concentration) to inhibit fungal and bacterial growth during zebrafish development.

Optional: If using FP-expressing transgenic zebrafish, use a fluorescence stereomicroscope to screen for fluorescent embryos at a suitable developmental stage when FP expression is known to be observed. This procedure is preferentially done during embryonic stages to minimize rearing and feeding of unnecessary nontransgenic fish.

3.2 REARING ZEBRAFISH TO POSTEMBRYONIC STAGES IN PREPARATION FOR FLUORESCENT LIPID STAINING

1. [Days 2–6; duration 15–30 min/day] Continue to raise embryos/larvae at 28.5°C in 100 mm Petri dishes until 5 days postfertilization (dpf). During these first 5 days of development, check daily for, and remove, dead embryos using a wide-bore Pasteur pipette and pipette pump (dead embryos are typically white in appearance). In addition, replace ~50% of water with fresh system water once every 2–3 days using a plastic transfer pipette. Zebrafish do not need exogenous nutrition until 5 dpf, therefore do not feed during this period.
2. [Day 6; static tank stage; duration ~15 min] At 5 dpf, transfer ~40 larvae to 1 L of fresh system water contained within a clean 2-L tank and fitted with 425-μm mesh drainage plugs.
3. [Day 6 onwards; duration ~15 min/day] From 5 dpf, and once larvae are transferred to 2-L tanks, feeding can commence. It is routine procedure for laboratory zebrafish facilities to feed young larvae (typically 5–10 dpf) either a *Paramecia multimicronucleatum* diet (http://zfin.org/zf_info/zfbook/chapt3/3.3.html) and/or commercial powdered food. We have also found feeding each 2-L tank containing 20–40 fish with 0.5 mL of ~1000 brine shrimp/mL concentration once per day can aid with larval survival. Dead brine shrimp and debris collecting at the bottom of the tank should be removed every few days with a plastic transfer pipette.

4. [Day 9; duration ~15 min] At 8 dpf, add 1 L of fresh system water to the existing 1 L containing larvae in each 2-L tank.
5. [Day 12; low flow tank stage; duration ~15 min/day] At 12 dpf, the 2-L tanks are placed on slow running system water with fine mesh (600 μm) drainage plugs. It is important to clean the drainage plugs every day to ensure unobstructed water flow. Continue feeding each tank with 0.5 mL of ~1000 brine shrimp/mL concentration once per day.
6. [Day 16; duration 15 min/day] Increase strength of water flow from 15 dpf. It is important to continue swapping mesh drainage plugs for ones with a larger mesh (typically 1600 and 4000 μm can be used) concomitant with growth of larvae/juveniles. Continue feeding each tank with 0.5 mL of ~1000 brine shrimp/mL concentration once per day.
7. Zebrafish that are fed using this protocol begin storing neutral lipid in adipocytes from ~10 to 15 dpf (Flynn et al., 2009). However, once independent feeding initiates (5 dpf), subsequent larval and juvenile growth rates vary considerably. Physical measurements such as standard length (SL; defined as distance from snout tip to caudal peduncle) provide more accurate metrics of postembryonic zebrafish development and growth (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). AT development in wild-type zebrafish is robustly correlated with SL (Imrie & Sadler, 2010; McMenamin et al., 2013). SL can be measured directly on live larvae/juveniles using a stereomicroscope equipped with an eyepiece graticule. Alternatively, the specimen can be imaged on a stereomicroscope using known magnification and subsequently measured using suitable image analysis software (e.g., ImageJ or Adobe Photoshop).
8. [Day 16 onwards; duration 5 min] To undertake a lipid stain at a selected timepoint, larvae/juveniles must be transferred to a smaller vessel such as a 15-mL conical tube. Larval and juvenile zebrafish are very delicate; therefore, during this step, it is essential to handle them with care. To transfer larvae/juveniles from 2-L tanks to a suitable vessel, fill a clean 2-L tank with system water. Then place a brine shrimp net into the freshly prepared tank of system water, and gently pour your larvae/juvenile sample into the net partially submerged within the freshly prepared tank. Pour carefully so that the larvae are not buffeted by water, as vigorous pouring will damage sample. Individually remove each larvae/juvenile from the net while partially submerged in the tank of system water using a plastic transfer pipette, and place in a 15-mL conical tube filled with an appropriate volume of fresh system water.

3.3 STAINING LIVE ZEBRAFISH LARVAE/JUVENILES WITH FLUORESCENT LIPOPHILIC DYES

A distinguishing feature of white adipocytes in fish as well as mammals is the presence of large cytoplasmic neutral LDs that consist largely of TG (Hashemi & Goodman, 2015). As described below, these characteristic adipocyte organelles can be unambiguously labeled in

live zebrafish using any of several commercially available fluorescent lipophilic probes (see Table 1).

1. [Duration 5 min] Wash unanesthetized larvae/juveniles in fresh system water three times at room temperature. This is accomplished by removing 80% of the system water from the 15-mL conical tube with a transfer pipette before adding fresh system water. This step removes debris included with larvae. After final wash, adjust volume of each 15-mL conical tube containing zebrafish up to 5 mL with fresh system water.
2. [Duration 5 min] Add lipid probe, at appropriate concentration (see Table 1), to 5 mL fresh system water containing larvae/juveniles. Lipid probe dissolved in DMSO can be added directly to system water. However, lipid probe dissolved in chloroform:MeOH must first be dried by chloroform:MeOH evaporation then resuspended in 100% EtOH (see Methods section). It is important to minimize the volume of 100% EtOH added to system water containing specimen, therefore we typically add 10 μ L to 5 mL system water containing fish.
3. The experimental procedure for zebrafish LD staining varies dependent on lipid probe used. Based on FLDs that we have used for staining LDs in zebrafish (see Table 1), we have devised two main protocols (see Fig. 2 for details). Protocol 1 should be followed for BODIPY dyes (both nonpolar and fatty acid/cholesteryl conjugates) as these require extensive wash steps to reduce background staining. Protocol 2 should be followed for Nile Red and HCS LipidTOX stains as these dyes do not require a wash step. Subsequent to the lipid staining protocol, all specimens are imaged using standard techniques regardless of protocol followed (see Sections 3.4 and 3.5).

Optional: It is common for ingested food and bile within the intestine to undergo autofluorescence. To reduce these unwanted fluorescence signals, fish can be starved overnight to “clear” the intestine of food before beginning the lipid stain.

3.4 IN VIVO IMAGING OF NEUTRAL LIPID ON A FLUORESCENCE STEREOMICROSCOPE

Imaging fluorescently labeled neutral lipid on a stereomicroscope allows for relatively high-throughput analysis of whole animal fat deposition (Fig. 2). Using the following imaging procedure, it is possible to acquire multiple images of distinct adipose depots in an individual fish within 5 min. Therefore, stereoscopic imaging of fluorescently stained zebrafish ATs is suitably quick for use as a viable phenotyping assay during chemical or genetic screens for factors that influence lipid storage in ATs.

To image and measure stained zebrafish, 1 $\times$ tricaine is used as a standard anesthetic in zebrafish research. Embryonic and larval zebrafish are particularly amenable to dosing with 1 $\times$ Tricaine, and recovery after 72 h of anesthesia is commonplace. However, older larval (larger than ~7 mm SL), juvenile and adult zebrafish are more difficult to recover after Tricaine anesthesia. Presumably this is due to increasing requirement for gill respiration as the zebrafish grows due to increased body size and epidermal thickness, combined with the

absence of active gill respiration while under Tricaine anesthesia. The consequence of increased sensitivity to Tricaine anesthesia is higher rates of death. We try to minimize exposure to Tricaine by imaging as quickly as possible and increasing animal number to compensate for any death that might occur.

1. Fill a 100 mm Petri dish with 30 mL of fresh system water and add 1.25 mL of 24×Tricaine.
2. Place a 3 × 3 mm drop of 4% methyl cellulose at the center of a 100 mm Petri dish lid. Cover the 4% methyl cellulose drop with system water containing 1×Tricaine.
3. Transfer stained unanesthetized fish from 15 mL conical tube to fish water containing Tricaine. Allow fish to sit in Tricaine-containing system water for ~5 min, or until fish has symptoms of being under anesthesia (i.e., belly up swimming, reducing gill respiration). Do not keep fish in Tricaine for longer than required as this may impede recovery from anesthesia.
4. Immediately transfer anesthetized fish to Petri dish lid containing 4% methyl cellulose droplet. Gently position tail of larvae/juvenile in the methyl cellulose and orientate appropriately using a metal dissection probe. Do not completely embed specimen in 4% methyl cellulose as this increases likelihood of damaging fish when subsequently releasing it. In zebrafish, neutral lipid within adipocytes is first deposited in association with the pancreas at ~4.4 mm SL, which is asymmetrically located on the right-hand side of the visceral cavity larvae/juveniles (Fig. 1A) (Flynn et al., 2009). Therefore, it is usually important to orientate specimen so that right-hand side is observable to the microscope objective.
5. Once larvae/juveniles are positioned correctly, measure SL using an eyepiece graticule.

Optional: The zebrafish pigment pattern can obscure imaging of adipose depots. Before imaging, confounding effects of the zebrafish pigment pattern can be reduced by treating animals with 10 mg/mL epinephrine for 5 min to contract melanosomes (Rawls & Johnson, 2003). This step is not optimal as epinephrine is known to stimulate lipolysis in adipocytes (Fain & Garcija-Sainz, 1983); however, our imaging procedure is sufficiently brief to prevent any salient effect on fat storage. Furthermore, for imaging visceral/pancreatic AT, melanin is usually not a problem during larval and early juvenile stages (i.e., through ~8 mm SL). Alternative solutions include using zebrafish mutants that fail to develop pigment cells; however, the potential effects of these mutations on adipose development and function have not been explored. Although inhibition of melanin synthesis during zebrafish development can be achieved by treatment with phenylthiourea, this is not practical for fish kept in tanks maintained on flowing recirculating water.

3.5 IN VIVO IMAGING OF NEUTRAL LIPID ON A CONFOCAL MICROSCOPE

Although considerably more time-consuming than stereomicroscope analysis, imaging fluorescently stained neutral lipid by confocal microscopy provides much greater resolution. Furthermore, if FP transgenic lines are used, exact colocalization of adipocyte LDs with fluorescent cell types of interest is achievable (Minchin et al., 2015). The following protocol takes ~35 min per zebrafish imaged (based on a 15 min Z-stack); however, exact timing will vary depending on length of confocal scan taken.

1. Anesthetize fish in Tricaine (see Section 3.4).
2. Measure SL of each fish to be imaged (see list 7 in Section 3.2).
3. Thaw 1% LMP agarose aliquots by placing 1 mL aliquot at 65°C until completely melted. Cool LMP agarose to 42°C using a heat block or water bath for 1 h until needed.

Note: For confocal imaging there are multiple methods for stably mounting zebrafish in 1% LMP agarose during imaging. Depending on microscope design (inverted or upright) and the type of objective to be used (air, immersion, or dipping) we have found the following methods for mounting to be most successful (see Fig. 3).

4. Upright confocal microscopes. It is preferential to use a water dipping objective. First, deposit a 3 × 3 mm droplet of 4% methyl cellulose in the center of a 30 mm Petri dish. Anesthetize larvae/juveniles in 1× Tricaine (see Section 3.4), transfer to methyl cellulose droplet, and roughly orientate. Remove excess system water with plastic transfer pipette and replace with 1% LMP agarose cooled to 42°C. Using a stereoscope quickly orientate sample into correct position with a metal dissecting probe. Continuously observe specimen as LMP solidifies to ensure correct positioning. It is important for specimen to be only lightly covered by LMP agarose. If too much LMP is added, it will prevent objective focusing to deep regions within zebrafish AT. Once LMP agarose is solidified, add ~2 mL of 1× Tricaine diluted in system water to the Petri dish to keep fish anesthetized.
5. Inverted confocal microscopes. It is common to use either air or immersion objectives. We mount the specimen in 30-mm Petri dish with glass cover slip as base. When using this method it is important to orientate specimen as close to cover slip as possible. Anesthetize zebrafish in 1× Tricaine (see Section 3.4) and place on the glass cover slip within the 30 mm Petri dish. Remove excess system water carried over from transfer of specimen. Quickly add 1% LMP agarose cooled to 42°C to fish and orientate into correct position using a metal dissection probe. Continuously observe orientation of the fish as LMP solidifies to ensure correct positioning. Once LMP agarose is solidified, add ~2 mL of 1% Tricaine diluted in system water to the Petri dish.
6. Table 1 contains excitation and emission information for each fluorescent lipid stain used to visualize neutral lipid. Due to the size of late larvae/juveniles, best

results are obtained using 10× or 20× objectives that have large working distances but a numerical aperture of ~1.

3.6 RECOVERY OF SAMPLE AFTER IMAGING OF FLUORESCENT NEUTRAL LIPID

1. Zebrafish are amenable to longitudinal analyses of fat storage within individual fish (Flynn et al., 2009; McMenamin et al., 2013). Therefore, once imaging has been completed, it is often necessary to recover larvae and allow development to proceed. Under a dissecting microscope gently cut LMP agarose away from tail of larvae with a metal dissection probe. We find it is easier to recover fish from 1% LMP agarose if it is first immersed in fresh system water. Once tail is free, it may be possible to release larvae by gently squeezing clean system water over the specimen with a plastic transfer pipette. If necessary, carefully remove more of LMP agarose from anterior regions until larvae are released.
2. Once larvae are free and recovered from anesthesia, individually house each fish in a well of a 24-well plate filled with 1 mL system water to keep record of subsequent larvae/juvenile growth during longitudinal analysis. It is necessary to change 80% of system water within each well daily and to feed ~30 live brine shrimp per well per day.

3.7 GUIDELINES FOR ANALYZING REGIONAL ZEBRAFISH ADIPOSE TISSUES

As described above, the ability to image the regional deposition of AT in whole animals has significant potential for investigating genetic and environmental factors that regulate AT distribution and disease susceptibility. Zebrafish are particularly amenable to whole-animal imaging of AT regionality (Fig. 1); therefore, we recently established a preliminary classification system and nomenclature for zebrafish ATs to promote experimental reliability and precision. Our proposed classification system is based on an existing system used to classify human ATs (Table 2) (Shen et al., 2003), and first divides total AT into “internal” or “subcutaneous” compartments (Fig. 4). Internal AT is subsequently divided into visceral (VAT) and nonvisceral (NVAT) ATs (Fig. 4). Subcutaneous ATs are more numerous and deposited throughout the body (Fig. 4). We categorized SAT into cranial, trunk, and appendicular deposits (Fig. 4). Table 2 and Fig. 4 provide a detailed overview of the zebrafish adipose classification system. Using the new classification system it is possible to quantify the area of each AT within an FLD-labeled using the fluorescence stereomicroscopy techniques outlined in Section 3.4. Briefly, individual ATs can be segmented using fluorescence thresholding to create an ROI that accurately represents the 2D area of an AT (Fig. 4A). In turn it has been shown that AT area, as assessed by FLD staining and stereomicroscopy, is an accurate measure of lipid content (Tingaud-Sequeira, 2011 #3). The majority of zebrafish ATs do not touch or merge with another AT; therefore, pixel intensity-based thresholding is the simplest and quickest segmentation method. For ATs that do touch (e.g., PVAT and AVAT) an intersecting boundary was defined by a straight line connecting the two AT extremes. Using this methodology it is possible to obtain a picture of lipid deposition within zebrafish ATs across a large developmental timeframe (Fig. 5).

4. SUMMARY

Investigation of white ATs has only recently been initiated in the zebrafish, and consequently we have a relatively limited knowledge of AT development and physiology in this important vertebrate model system. However, the amenability of the zebrafish to high resolution in vivo imaging presents exciting opportunities to address longstanding questions about AT formation and function which have been difficult to resolve using available mammalian models. Moreover, systematic genetic and chemical tests in the zebrafish model could be used to identify novel factors regulating distinct aspects of AT biology.

Here we have provided detailed methods for in vivo imaging of zebrafish ATs to support the use of the zebrafish as a model for AT research. We anticipate that the fluorescence stereomicroscopy methods presented here will be useful for rapid phenotypic assessments required for genetic and chemical screens, while the confocal microscopy methods will facilitate high-resolution analysis of cellular and molecular events within ATs. Furthermore, we expect that these methods will also be generally applicable to in vivo imaging of ATs in other fish species. Deployment of these methods in the zebrafish system will be enriched by the use of existing and forthcoming zebrafish lines expressing FPs and other transgenes in adipocyte lineages and other cellular constituents of ATs. Use of zebrafish as a model for AT biology will also be enhanced by identification of genetic alterations, dietary and environmental manipulations, and chemicals that modify zebrafish AT formation and function.

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FIGURE 1. Fluorescent lipophilic dyes reveal lipid droplet accumulation in live zebrafish
 Live zebrafish stained with Nile Red (yellow) and imaged by fluorescence stereomicroscopy using the protocol described in Section 3.4. The standard length in millimeter of individual zebrafish is indicated in the bottom left corner of each image and the accompanying postembryonic stage is indicated in *parenthesis* as defined by Parichy et al. (2009). *Arrows* indicate the increasing complexity of regional lipid deposits in the zebrafish head.

FIGURE 2. Flow diagram depicting protocols for staining zebrafish with fluorescent lipophilic dyes

Protocol 1 includes wash steps and should be used for BODIPY dyes. Protocol 2 does not contain wash steps and should be used for HCS LipidTOX and Nile Red dyes. See Table 1 for information on lipophilic dyes.

FIGURE 3. Schematic illustrating stable mounting procedures for regular and inverted microscopes

(A) When imaging using an upright objective, a 30 mm Petri dish is used (step 1), and 1% regular agarose is used to fill in around the edges of the Petri dish (step 2). 4% methyl cellulose is then placed in the center of Petri dish (step 3), before the anesthetized animal is placed in 4% methyl cellulose and orientated (step 4). Once specimen is correctly orientated, it is covered with 1% low melting point (LMP) agarose and allowed to solidify (step 5). (B) For imaging using an inverted objective, a 30-mm Petri dish with a fitted glass cover slip as a base is used (step 1). Anesthetized zebrafish are placed onto the glass cover slip base (step 2) and excess liquid is removed. The specimen is then covered with 1% LMP agarose (step 3) and quickly orientated while agarose is solidifying (step 4). For both methods, 2 mL of system water, containing 1× Tricaine, is added to specimen. When undertaking imaging using the upright objective method, be sure to use a water dipping objective.

FIGURE 4. Classification of regional adipose tissues (ATs) in zebrafish
(A) Schematic illustrating the experimental procedure used to identify 34 regionally distinct zebrafish ATs. (B) Circular graph illustrating the relationship between the 34 regionally distinct zebrafish ATs. Acronyms are defined in Table 2. (C). Schematic illustrating the anatomical location of zebrafish regional ATs. Circular graphs correspond to (B) and acronyms are defined in Table 2.

FIGURE 5. Box plots depicting the growth in area of zebrafish adipose tissues (ATs) across a range of postembryonic stages
Box plots depicting the growth of total AT (A), internal AT (B), and subcutaneous AT (C) across postembryonic stages. The stages are defined in Parichy et al. (2009).

Table 1

Lipophilic Fluorescent Dyes for Staining Lipid Droplets in Zebrafish

Color	Name	IUPAC Name	Cat. No. ^a	Solvent	Stock Conc.	Working Conc. ^b	Absorption/Emission Maxima (nm)	Additional Notes ^c
Green	BODIPY 500/510	4,4-Difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanole acid	D3823	Chloroform: MeOH	1 mg/mL	0.5 mg/mL (2000 ×)	500/510 (use filter sets appropriate for Alexa Fluor 488)	Very bright lipid droplet (LD) stain, weak gall bladder stain
Green	BODIPY 505/515	4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene	D3921	DMSO	1 mg/mL	1 µg/mL (1000 ×)	505/515 (use filter sets appropriate for Alexa Fluor 488)	Very bright LD stain, bright gall bladder stain, significant fluorescence emission in red channel after 488 nm laser excitation
Green	BODIPY FL CS	4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanole acid	D3834	Chloroform: MeOH	0.5 mg/mL	0.25 mg/mL (2000 ×)	503/512 (use filter sets appropriate for Alexa Fluor 488)	Weak LD stain, bright gall bladder stain, bright intestine stain
Green	NBT—cholesterol	22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesten-3β-ol	N1148	Chloroform: MeOH	5 µg/mL	0.5 µg/mL (10,000 ×)	Absorption/emission maxima is dependent on solvent and environment. However, we use filter sets appropriate for Alexa Fluor 488	Bright LD stain, weak gall bladder stain
Yellow	BODIPY 530/550	4,4-Difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanole acid	D3832	Chloroform: MeOH	1 mg/mL	10 µg/mL (100 ×)	530/550 (use filter sets appropriate for Alexa Fluor 532)	Very weak LD stain, high non-LD background
Yellow-orange	Nile Red	9-Diethylamino-5H-benz[α]phenoxazine-5-one	N1142	Acetone	1.25 mg/mL	0.5 µg/mL	510/580 (excite with Argon 514 nm laser, collect emission with a long pass 530 filter) ^d	Very bright LD stain, red phospholipid background stain
Orange	BODIPY 558/568	4,4-Difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-dodecanole acid	D3835	Chloroform: MeOH	1 mg/mL	2 µg/mL (500 ×)	558/568 (use filter sets appropriate for Alexa Fluor 546)	Very bright LD stain, very bright non-LD background
Red-orange	Cholesteryl BODIPY 576/589	Cholesteryl 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoate	C12681	Chloroform: MeOH	1 mg/mL	10 µg/mL (100 ×)	576/589 (use filter sets appropriate for Alexa Fluor 568)	Weak LD stain
Red	HCS LipidTOX red	—	H34476	DMSO	—	(5000 ×)	577/609 (use filter sets appropriate for Alexa Fluor 594 or Texas Red)	Very bright LD stain, bright gall bladder stain, zero non-LD background
Fair-red	HCS LipidTOX deep red	—	H34477	DMSO	—	(5000 ×)	637/655 (use filter sets appropriate for Alexa Fluor 647 or Cy5 dye)	Very bright LD stain, bright gall bladder stain, zero non-LD background

^a All catalog numbers (cat. no.) are from Invitrogen.

^b Dilution of stock solution to achieve working concentration is in parentheses.

^c Staining patterns correspond to wild-type fish raised under the husbandry protocols described here. These staining patterns could potentially be altered as a function of fish genotype, dietary status, and other exposures.

^d Nile Red bound to phospholipid bilayer has absorption/emission maxima of ~550/640. Alexa Fluor dyes are from Invitrogen.

Table 2

Classification System for Zebrafish Adipose Tissues (ATs)

AT Classification		Acronym	AT Appearance (Standard Length (SL) mm) ^a
Internal	Visceral	IAT	4.4
		VAT	4.4
	Cardiac	CVAT	9.7
		aCVAT	9.7
	Pancreatic	pCVAT	12.5
		PVAT	4.4
	Abdominal	AVAT	5.5
		RVAT	7.9
	Nonvisceral	NVAT	8.7
		POS	8.9
Subcutaneous	Appendicular	dPOS	10
		cPOS	9.1
		vPOS	11.5
		IM	10.2
		cIM	10.1
		dIM	n.d.
		vIM	n.d.
		SAT	6.6
		APPSAT	9.3
		CFRSAT	10.8
	Pectoral fin	DFRSAT	11.5
		AFRSAT	9.7
		AFCSAT	10
		PELSAT	n.d.
		PECSAT	10.2
		aPECSAT	12.5
		pPECSAT	11.8
		lPECSAT	11.5

AT Classification			Acronym	AT Appearance (Standard Length (SL) mm) ^a
Cranial	Ocular	Opercular	CSAT	6.6
			OCU	7.2
			OPC	9.5
Truncal	Hyoid	Dorsal	dOPC	10.3
		Ventral	vOPC	9.5
		Basilhyoid	HYD	6.9
			BHD	7.3
			CHD	7.7
	Lateral	Ceratohyoid	UHD	n.d.
		Urohyoid	TSAT	8.2
		Dorsal	LSAT	8.2
			DSAT	9.5
			aDSAT	10
	Ventral	Anterior	pDSAT	9.5
		Posterior	VSAT	9.8
		Abdominal	ASAT	10.5

^aThe SL at which zebrafish ATs first appear ($P > 0.5$) was identified using logistic regression within a cohort of 362 Ekkwill wild-type fish.